IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR LETTERS PATENT OF THE UNITED STATES

TITLE:

METHOD OF DETERMINING THE
SEQUENCE OF NUCLEIC ACIDS
EMPLOYING SOLID-PHASE
PARTICLES CARRYING
TRANSPONDERS

INVENTOR:

WLODEK MANDECKI

ATTORNEYS:

Robert W. Stevenson
Curt J. Whitenack
WILLIAN BRINKS HOFER
    GILSON & LIONE
P.O. Box 10395
Chicago, Illinois 60611
(312) 321-4200

BACKGROUND OF THE INVENTION

This invention relates to materials and methods for determining the sequence of unknown or target nucleic acids, and more specifically to materials and methods for determining the sequence of target nucleic acids using an electronically-indexed solid phase, with transponders associated with the solid phase particles.

A high throughput method for ascertaining the sequence of sample nucleic acids is sequencing by hybridization (SBH). In that method, a large number of oligonucleotide probes is allowed to interact with the nucleic acid molecules in a sample, and a detection system is provided to determine whether individual oligonucleotides have annealed to the template. Two basic designs have been described. In one, the oligonucleotide probes are arranged in a two-dimensional array on the surface of a membrane, filter, VLSI chip, or the like. In the other, the array on the membrane is formed with a large number of sample DNA sequences, and each membrane is subjected to a series of hybridization steps with different oligonucleotide probes. A label used to monitor the binding, either a radioactive isotope or a fluorophore, is carried on the sample DNA or on the oligonucleotide probe. The sequence is derived from coordinates of spots showing a high level of the label deposition on the two-dimensional arrays.

Conventional SBH methods are limited by difficulties related to preparation of arrays of DNA, non-specific annealing of DNAs, the need for special instrumentation to read the data and the automation of the process and data analysis. In SBH, the sequence is determined by defining the two-dimensional coordinates of relevant dots in the array formed by the deposited DNA molecules. In the present invention, a partial or the complete sequence of the DNA molecule is determined by decoding the electronic memory elements associated with DNA probes of known sequence.

An advantage of the present invention over conventional sequencing methods is that it is extremely fast,

because the sequence is deduced from a series of readings of
digitally-stored sequences in the transponder, rather than
from a series of measurements of a chemical or physical
property of DNA, or the location of DNA in an array. The
5   method of this invention is referred to hereinafter as digital
sequencing.

SUMMARY OF THE INVENTION

The present invention overcomes the problems of
10   conventional sequencing methods by employing solid phase
particles having a transponder associated with each particle.
The particles carry oligonucleotide probes attached to their
surface, and the sequence of the oligonucleotide is encoded on
a memory element on the transponder. The oligonucleotide
15   probes correspond to subsequences believed to exist in the
target sequence.

To determine the sequence of sample, or "target"
DNA, the target DNA of unknown sequence is labeled with a
fluorophore and combined with transponder particles carrying
20   known oligonucleotides under annealing conditions. The
transponders are analyzed to detect the fluorescence or color
originating from a label that indicates that target DNA has
bound to the probe attached to the surface of the transponder,
and the information stored electronically in the transponder
25   is decoded. Dedicated sequence analysis software may then be
used to determine the complete or partial sequence of the DNA
target.

In one aspect, the present invention provides a
solid phase particle for use in determining the sequence of
30   nucleic acids, comprising a solid phase particle having a
transponder, and an oligonucleotide probe having a known
sequence attached to an outer surface of the particle.

In another aspect, the present invention provides a
method of determining the sequence of sample nucleic acids,
35   comprising the steps of employing solid phase particles with
transponders.

In another aspect, the invention provides a kit for

determining the sequence of unknown nucleic acids, comprising an assay vessel, and a set of solid phase particles having transponders, and a different oligonucleotide attached to the surface of the probe.

5

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of a sequencing procedure of this invention.

FIG. 2 is a cross-sectional view of a solid phase particle with a transponder, and a primary layer of biomolecules bound to a surface thereof.

FIG. 3 is a schematic diagram of the signal pathway for encoding and decoding data on the transponders of the solid phase.

FIG. 4 is a schematic representation of a miniature transponder.

FIG. 5 is a plan view of a miniature transponder.

FIG. 6 is a plan view of a transport system/analytical instrument for implementing the present invention.

FIG. 7 is a plan view of a modified flow cytometer for high speed analysis of solid phase particles of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Figure 1 depicts a simple sequencing procedure of the current invention. A solid phase particle 10 with a transponder 12 is derivatized by attaching an oligonucleotide probe 14 of known sequence to the outer surface 16 of the particle 10. The transponder 12 is encoded with an index number that indicates the sequence of the oligonucleotide probe 14. The particle 10 is immersed in a solution containing labelled target DNA 11, and the solution is heated to cause DNA to dissociate, then cooled to allow the DNA to anneal, resulting in target DNA 11 annealing to the probe 14. The transponder 12 is analyzed to detect the fluorescence of any target DNA 11 bound to the transponder 12, and the

transponder 12 is decoded using a read/write scanner device (not shown). In practice a group of transponders, each carrying a different probe and each encoded with the sequence of that probe would be used.

The target DNA is preferably pre-treated by digestion with an appropriate restriction endonuclease, or fragmented by digesting with DNase I, to yield relatively short (preferably 10 nt to 100 nt) preferably single-stranded DNA. Pretreatment may also involve a conversion of DNA to RNA by cloning and in vitro transcription, followed by a partial hydrolysis of RNA (if necessary), or a generation of a DNA fragment by PCR, and the latter can be coupled with labeling of DNA with a fluorophore. DNA provided for sequencing may already be in the preferred form. If, however, the DNA is in the form of a long double-stranded DNA molecule, a long single-stranded DNA molecule, a closed circular DNA molecule, or a nicked circular double-stranded DNA molecule, the DNA must be pre-treated.

Labelling sample DNA with a fluorogenic hapten before annealing is a practical, although not always necessary, step in digital sequencing. Restriction fragments with the 5'-protruding end can be labeled in a chain extension reaction with a DNA polymerase utilizing one or more of dNTPs derivatized with a fluorogenic hapten. 3'-ends of DNA molecules can be labeled in an enzymatic reaction employing a terminal deoxynucleotidyl transferase (TdT) and dNTP derivatized with a fluorogenic hapten. In some sandwich-type applications, the fluorogenic hapten can be replaced with any hapten (e.g. biotin) for which an antibody (or a binding partner) is available, the goal being to detect the binding of labeled DNA to the transponders through a sandwich configuration involving an anti-hapten antibody and a secondary anti-antibody antibody conjugated to an enzyme, said enzyme catalyzing a reaction with a precipitating fluorogenic substrate.

Several types of nucleic acid can be immobilized on the transponders. They include DNA, RNA and modifications

thereof, such as protein-nucleic acid (PNA) molecules. It is preferred that the immobilized nucleic acids are single-stranded. The length of the immobilized nucleic acids can vary in different implementations of the digital sequencing, the requirement being that the length should be sufficient to provide a desired level of binding specificity to the target DNA. In one embodiment of this invention, the oligonucleotide probes correspond to subsequences that are believed to exist in the target nucleic acid sequence.

There are several methods to immobilize nucleic acids on the solid phase particles of this invention, including the conjugation of oligonucleotides to the transponders, or the direct chemical synthesis of the oligonucleotide on the transponders. Combinatorial synthesis is a preferred method of direct chemical synthesis, and involves up to four independent condensations of a four different nucleosides (A, C, G and T) on a large number of transponders in four vessels. The transponders are divided into four pools, and each pool is reacted with a different nucleoside. After each condensation, the transponders in the four pools are encoded with a symbol indicating the nucleoside used in the condensation. The four transponder pools are then combined and redistributed into four vessels, and the process is repeated as many times as necessary and practical. The net result of combinatorial synthesis on transponders is that the transponders are derivatized with different oligonucleotides, and the sequence of the oligonucleotide is encoded in the transponder.

The derivatized transponders and the target DNA are kept in a single vessel in an appropriate buffer. The volume of the buffer must be sufficient to completely immerse the transponders in the buffer. An appropriate buffer for the annealing reaction is phosphate-buffered saline (PBS), but many other buffers are suitable, as is well-known by those of ordinary skill in this art. The vessel is heated to a temperature typically in the range of 60°-100°C. The temperature should be sufficient to allow for melting of

double-stranded DNA that might exist in the vessel into the single-stranded form. The vessel is then slowly cooled to a temperature below the melting temperature for the sequences immobilized on the transponder, which typically is in the range of 0°-60°C, and is often room temperature. The transponders are then washed thoroughly several times to remove unbound target DNA.

If the target DNA was fluorescence-labeled before annealing, no post-annealing treatment is needed, and the transponders can be subjected to decoding, and the fluorescence of the surface can be measured. Alternatively, if the target DNA was not labeled, fluorescence-labeling must be done after annealing. Either target DNA (associated with the oligonucleotide bound to the transponder) can be labeled, or the oligonucleotide probe on the transponder (but only those that are associated with the target DNA) can be labeled. A method suitable for both approaches is chain extension utilizing a DNA polymerase.

Chain extension labeling procedures can differ with regard to: (a) the type of the fluorophore-tagged nucleotide used for the labeling, or (b) the number of different fluorescent nucleotides used in the extension reaction (choice of the adenine, cytosine, guanine or thymine derivatives). As to the type of nucleotide used, the nucleotide can be either of the deoxy type, or the dideoxy type. More than one labeled deoxy nucleotide can be incorporated into the extended portion of the chain, but the incorporation of only one dideoxy nucleotide is attainable, since it prevents further chain extension.

The labeling can be combined with a fluorescence detection method for transponders which allows for distinguishing the type of the fluorescence label used by identifying the maximum emission wavelength of the fluorophore, similar to the implementation in automated sequencing on Applied Biosystems sequencers. The use of dideoxy nucleotides in digital sequencing, therefore, offers the ability to identify the residue immediately proximal to

the 3' end of either target DNA, or the oligonucleotide on the transponder, depending on the approach used.

In a preferred embodiment of the labeling procedure in the presence of four fluorophore-labeled dideoxy nucleotide triphosphates (ddATP,ddCTP,ddGTP and ddTTP) the primers attached to the transponder that annealed to the target are extended by one nucleotide residue by a DNA polymerase. The type of the incorporated residue (A, C, G or T) is determined by the sequence of the target. Therefore, the wavelength at which the maximum intensity of fluorescence is observed indicates the residue type. After the extension step, the particles are passed through a fluorometer capable of discriminating between four wavelengths of emitted light. In this approach, more information is obtained than in the basic implementation of the method. As previously, the presence of the fluorescence is an indication that the annealing took place, but now the wavelength of the fluorescence additionally identifies the target residue that is immediately downstream from the primer (i.e. close to the 3' end of the primer).

In an alternative version of the above procedure, only one fluorophore is used in the reaction, but four separate extension reactions are performed in four separate vessels employing one of four ddNTPs in each vessel. After the reaction and appropriate washes, the particles from the four vessels are mixed together, and the remainder of the procedure is carried through as presented above. In this case, however, an additional encoding step of the particle's memory is needed to provide the information about the type of nucleotide used in the extension reaction for the given transponder.

Fig. 2 depicts a solid phase particle for use in this invention. The particle 10 is derivatized by attaching an oligonucleotide probe 14 of known sequence to a surface 16 of the particle 10. A transponder 12 is associated with the particle 10. The transponder 12 is encoded with an index number that indicates the sequence of the probe 14. The transponder may be pre-programmed by the manufacturer, or it

may be encoded by the user, using a scanner read/write device.

A transponder is a radio transmitter-receiver activated for transmission of data by reception of a predetermined signal, and may also be referred to as a microtransponder, radiotransponder, radio tag, transceiver, etc. The signal comes from a dedicated scanner, which also receives and processes the data sent by the transponder in response to the signal. The scanner function can be combined with the write function, i.e. the process of encoding the data on the transponder. Such a combination instrument is called a scanner read/write device. An advantage of the transponder-scanner system stems from the fact the two units are not physically connected by wire, but are coupled inductively, i.e. by the use of electromagnetic radiation, typically in the range from 5-1,000 kHz, but also up to 1 GHz and higher.

Figure 3 is a flow chart illustrating the communication between the transponder 12 and a remote scanner read/write device 18. The transponder 12 associated with the solid phase beads 10 is encoded with data sent by electromagnetic waves from a remote scanner read/write device 18. After the assay steps are completed, the beads 10 are analyzed to detect the presence of a label indicative of binding of analyte and those that show the presence of the label are decoded. The scanner 18 sends a signal to the transponder 12. In response to the signal, the transponder 12 transmits the encoded data to the scanner 18.

Some transponders similar to those used in this invention are available commercially. Bio Medic Data Systems Inc. (BMDS, 255 West Spring Valley Ave., Maywood, New Jersey) manufactures a programmable transponder for use in laboratory animal identification. The transponder is implanted in the body of an animal, such as a mouse, and is glass-encapsulated to protect the electronics inside the transponder from the environment. One of the transponders manufactured by this corporation, model# IPTT-100, has dimensions of 14 x 2.2 x 2.2 mm and weighs 120 mg. The transponder is

user-programmable with up to 16 alphanumeric characters, the
16th letter programmable independently of the other 15
letters, and has a built-in temperature sensor as well. The
electronic animal monitoring system (ELAMS) includes also a
scanner read/write system to encode or read data on/from the
transponder. The construction of the transponder and scanner
is described in U.S. Patent Nos. 5,250,944, 5,252,962 and
5,262,772, the disclosures of which are incorporated herein by
reference. Other similar transponder-scanner systems include
a multi-memory electronic identification tag (U.S. Patent
5,257,011) manufactured by AVID Corporation (Norco, CA) and a
system made by TEMIC-Telefunken (Eching, Germany). AVID's
transponder has dimensions of 1 mm x 1 mm x 11 mm, and can
encode 96 bits of information, programmed by the user. The
present invention can be practiced with different
transponders, which might be of different dimensions and have
different electronic memory capacity.

The commercially available transponders are
relatively large in size. The speed at which the transponders
may be decoded is limited by the carrier frequency and the
method of transmitting the data. In typical signal
transmission schemes, the data are encoded by modulating
either the amplitude, frequency or phase of the carrier.
Depending on the modulation method chosen, compression
schemes, transmission environment, noise and other factors,
the rate of the signal transmission is within two orders of
magnitude of the carrier frequency. For example, a carrier
frequency of 1,000 Hz corresponds to rates of 10 to 100,000
bits per second (bps). At the rate 10,000 bps the
transmission of 100 bits will take 0.01 sec. The carrier
frequency can be several orders of magnitude higher than 1,000
Hz, so the transmission rates can be proportionally higher as
well.

Therefore, the limiting factor in the screening
process is the speed at which the transport mechanism carries
the transponders through the read window of the
fluorometer/scanner device. The rate of movement of small

particles or cells is 10^4-10^5 per second in state-of-the-art flow cytometers. A flow cytometer may be used to practice the present invention, if two conditions are met: (1) the transponders are small enough to pass through the flow

5   chamber, and (2) the design of the flow chamber of the flow cytometer is modified to include an antenna for collecting the electromagnetic radiation emitted by transponders.

A miniature transponder is depicted in Figs. 4 and 5. The source of the electrical power for the transponder 12a

10   is at least one photovoltaic cell 40 within the transponder 12a, illuminated by light, preferably from a laser (not shown). The same light also induces the fluorescence of the fluorogenic molecules immobilized on the surface of the transponder 12a. The transponder 12a includes a memory

15   element 42 that may be of the EEPROM type. The contents of the memory is converted from the digital form to the analog form by a Digital-to-Analog converter 44 mounted on the transponder 12a. The signal is amplified by an amplifier 46, mixed with the carrier signal produced by an oscillator 48,

20   and emitted to the outside of the transponder 12a by an antenna 50.

The contents of the transponder memory can be permanently encoded during the manufacturing process of the transponder, different batches of transponders being differently encoded.

25   Preferably, the memory of the transponder is user-programmable, and is encoded by the user just before, during, or just after the biological material is deposited on the surface of the transponder. A user-programmable transponder 12a must have the "write" feature enabled by the antenna 50,

30   amplifier 44 and the Analog-to-Digital converter 46 manufactured on the transponder 12a, as well as the dedicated scanner read/write device 27.

The advantages of the transponder of Figs. 4 and 5 are several-fold. First, the dimension of the transponder is

35   reduced, since most of the volume of current transponders is occupied by the solenoid. The design discussed above will enable the production of cubic transponders on the order of

0.01 to 1.0 mm along a side, preferably 0.05 to 0.2 mm.

Second, a large number of transponders can be manufactured on a single silicon wafer, and no further assembly would be required to attach the solenoid to the VLSI

5   chip. As depicted schematically in Fig. 5, a silicon wafer 60 is simply cut to yield active transponders 12a. Third, the transponder, according the new design, will not need the glass capsule as an enclosure, further reducing the size of the transponder. Silicone dioxide (SiO_2) would constitute a

10   significant portion of the surface of the transponder, and SiO_2 has chemical properties which are very similar to glass in terms of the feasibility of derivatization or immobilization of biomolecules. Alternatively, microtransponders may be coated with a variety of materials, including plastic, latex

15   and the like.

Finally, most important, the narrow focus of the beam of the laser light would enable only one transponder to be active at a time, significantly reducing the noise level. Advanced user-programmability is desirable as well, various

20   memory registers need to be addressable independently (writing in one register should not erase the contents of other registers).

Figure 6 shows the analytical instrumentation and transport system used in an embodiment of the present

25   invention. A quartz tube 20 is mounted in the readout window 22 of a fluorometer 24. The quartz tube 20 is connected to a metal funnel 26. The length of the quartz tube 20 is similar to the dimensions of the transponder 12. Transponders 12 are fed into the metal funnel 26, and pass from the funnel 26 into

30   the quartz tube 20, where the fluorescence is read by the fluorometer 24 and the transponder 12 is decoded by the scanner 27, and then exit through a metal tube 28 and are conducted to a collection vessel (not shown). The metal funnel 26 and metal tube 28 are made of metal shield

35   transponders 12 outside of the read window 22 by shielding from the electromagnetic signal from the scanner 27. This shielding prevents the scanner signal from reaching more than

one transponder 12, causing multiple transponders 12 to be decoded.

Minimal modification of the fluorometer 24 would be needed in the vicinity of the location that the tube occupies at the readout moment to allow for positioning of the transponder reading device. To assure compatibility with existing assays, the glass surrounding the transponder could be coated with plastic currently used to manufacture beads.

In a preferred design, depicted in Fig. 4, a metal coil antenna 30 is wrapped around the flow cell 32 of a flow cytometer 29. The transponders 12 pass through the flow cell 32, and are decoded by the scanner device 27. The signal carrying the data sent from the transponders 12 is amplified by a first amplifier 34 and processed by the scanning device 27. As the transponders 12 are decoded, fluorescence from the transponders 12 is detected and analyzed by the flow cytometer 29.

In one embodiment of the present invention, multiple passes of the same set of transponders through the detector are implemented. The temperature is incrementally increased at each pass, the purpose being to gradually increase the stringency of binding, and to reduce effects of non-specific binding of the target to the probes on the transponders. After the detection step is completed, the transponders can be reconditioned for further use in another experiment by stripping the biomolecular coating chemically, and clearing the memory. If the target DNA is labeled with a fluorophore, an alternative to chemical stripping is the extensive wash at high temperature to dissociate and remove non-covalently bound target DNA.

Upon completion of the analysis step and decoding of the transponders, the data is analyzed to determine the sequence by correlating the sequence of the oligonucleotide probe immobilized on the transponder and the fluorescence readout of the transponder carrying the probe. The type of data related to the fluorescence readout may vary depending of the procedures used. If the target DNA is labeled with the

fluorophore prior to annealing, or if the label is a single fluorophore, introduced in a chain extension reaction with a DNA polymerase after annealing, the fluorescence readout is simply the intensity of the fluorescence. Alternatively, if four ddNTPs are used with the purpose of identifying the nucleotide residue proximal to the 3' end of the probe or target DNA (depending on the design of the experiment), the fluorescence readout is both the intensity and the information about the wavelength of maximal fluorescence. The form of the data set is analogous to that yielded by sequencing by hybridization methods(SBH), and algorithms previously developed for SBH can also be used for digital sequencing (Drmanac R. et al., 1993, DNA Sequence Determination By Hybridization: A Strategy For Efficient Large-Scale Sequencing. Science 260, 1649-1652).

EXAMPLE 1

Immobilization Of DNA Probes On Transponders Using The Chemical Synthesis Of DNA

Nucleic acids can be covalently linked to glass by direct chemical synthesis on a glass support. To prepare the support, 5'-dimethoxytrityl thymidine is reacted with one equivalent of tolylene-2,6-diisocyanate in the presence of one equivalent of N-ethyldiisopropylamine as a catalyst in pyridine/1,2-dichloroethane to generate the monoisocyanate. The monoisocyanate is not isolated, but is added directly to the alkylamine glass support, i.e. the aminopropyltriethoxysilane-derivatized glass surface of the transponders. The procedure is described in detail in B.S. Sproat and D.M. Brown, A New Linkage For Solid Phase Synthesis Of Oligodeoxyribonucleotides, Nucleic Acids Res. 13, 2979-2987, 1985. Such thymidine-derivatized support containing the stable nucleoside-urethane linkage is directly used for the chemical synthesis of oligodeoxynucleotides using a manual synthesis protocol on sintered funnels as described before (Caruthers, M.H. et al., Deoxyoligonucleotide synthesis via the phosphoramidite method. In: Gene Amplification and Analysis, Vol III (T.S. Papas et al., Eds.). Elsevier/North

Holland, Amsterdam), using standard phosphoramidite-based DNA synthesis reagent. The thymidine-urethane linker is resistant to cleavage with base during the deprotection, and the resulting product is the deprotected oligonucleotide attached

5 to the glass surface of the transponder through the urethane-thymidilate linker.

EXAMPLE 2
Determination Of The Sequence Of A Three
Nucleotide Region Using AVID Transponders

10 The target DNA is a single-stranded 50-residue long oligodeoxynucleotide. It is obtained from a PCR reaction using a 5'-fluoresceinated oligonucleotide as one of the primers, after linearly amplifying DNA. DNA used in the experiment is purified after PCR. Most of the sequence of 47

15 nucleotides in the 50 nt target is known, except for a region consisting of three residues, residue numbers 18-20, marked as NNN in the figure below:

transponder-linker-GGTACTGCXXXACCTTCCA-3'

||||||| |||||||

20 3'-ACGTTAAGCCCAGTATGCCATACCATGACGNNNTGGAAGGTAGAGATACT-5'-fluor

50   40   30   20   10   1

The symbol "|" denotes the complementarity of nucleotides in

25 the two sequences. There are exactly 64 possible sequences for 5'-GGTACTGCXXXACCTTCCA, where X can be any nucleotide residue (A, C, G or T). 64 sets of AVID transponders are prepared, each set having a different oligonucleotide probe (upper sequence in the figure above) synthesized on the

30 surface of the transponder using standard phosphoramidite chemistry as described in Example 1. The linker has the $(dT)_{10}$ sequence to facilitate annealing. The transponders are electronically encoded with a number 1 through 64, the number corresponding to the sequence of the oligonucleotide bound to

35 the transponder. 64 transponders, each from a different set, are put into a 10 ml vessel. A solution containing the target (5 ml volume) is then added to the vessel, the contents of the vessel is heated to 95°C, cooled slowly over the period of 10

min to the experimentally predetermined wash temperature T_{wash}
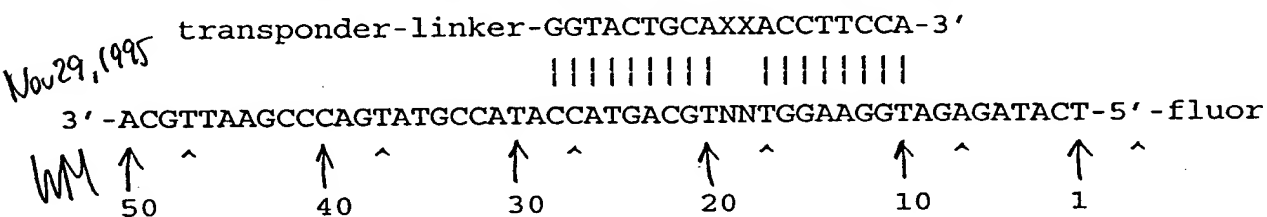(T_{wash} can range from room temperature to 60°C, data not shown).
After the annealing step, the transponders were extensively
washed at temperature T_{wash}, and then stored in a buffer at room
temperature.

Each of the 64 transponders was subjected to both
fluorescence measurement and electronic decoding of the tag.
As a result, each of 64 possible sequences was associated with
a number, fluorescence readout, that indicated whether the
template had annealed to the oligonucleotide on the
transponder, thus providing the information about the sequence
of the 3 nt region.

EXAMPLE 3

Determination Of The Sequence Of A Two Nucleotide Region Using
Combinatorial Oligonucleotide Synthesis On BMDS Transponders

The target was a single-stranded 50-residue long
oligonucleotide. It was obtained from a PCR reaction using a
5'-fluoresceinated oligonucleotide primer, after linearly
amplifying DNA. DNA used in the experiment is purified after
PCR. Most of the sequence of 48 nucleotides in the 50 nt
template was known, except for the region of two residues,
residue numbers 18-19, marked as NN in the figure below:

```
        transponder-linker-GGTACTGCAXXACCTTCCA-3'
                           |||||||||  |||||||||
    3'-ACGTTAAGCCCAGTATGCCATACCATGACGTNNTGGAAGGTAGAGATACT-5'-fluor
     WM ↑   ^     ↑   ^     ↑   ^     ↑   ^     ↑   ^     ↑   ^
        50        40        30        20        10        1
```

The symbol "|" denotes the complementarity of nucleotides in
the two sequences. The linker has the $(dT)_{10}$ sequence to
facilitate annealing. There are exactly 16 possible sequences
of oligonucleotides 5'-GGTACTGCAXXACCTTCCA, where X can be any
nucleotide residue (A, C, G or T). These sequences are
synthesized in a combinatorial fashion on 200 BMDS
transponders (14x2x2 mm, volume of each transponder is about
50 μl) as follows. First, the underivatized glass surface of
the transponders is treated as described in Example 1 to

introduce the functional hydroxy groups on the surface of
transponders. Then, the transponders are subjected to 18
rounds of solid phase oligonucleotide synthesis using standard
phosphoramidite chemistry to sequentially synthesize the
following residues: TTTTTTTTTTGGTACTGCA-3'. The transponders
are split into four groups of approximately equal numbers
(i.e. 50). Each of the four groups is subjected to a single
cycle of solid phase oligonucleotide synthesis with a
different nucleotide phosphoramidite. That is, transponders
from group 1 are derivatized with adenosine phosphoramidite
(A), group 2 - cytosine phosphoramidite (C), group 3 -
guanosine phosphoramidite (G), and group 4 - thymidine
phosphoramidite (T). The volume of each of the four
condensations is about 4 ml. After completing the first
cycle, the 4 groups are subjected to electronic encoding with
the residue abbreviations given above. Thus, the first
alphanumeric character indicates which condensation takes
place in the first cycle of combinatorial synthesis. Then,
all the transponders are pooled together and mixed thoroughly,
and then split into new four groups having approximately the
same number of transponders. These four new groups are
subjected to the second combinatorial coupling step. The
scheme for the second coupling is identical to that of the
first coupling. Electronic encoding after the second cycle
adds a second alphanumeric character, corresponding to the
nucleoside coupled in the second synthesis cycle. As a
result, each transponder is encoded with two alphanumeric
characters which identify the sequence of two nucleotide
residues at the 3' end of the oligonucleotide attached to the
surface of the transponder. The transponders having
oligonucleotides coupled to their surfaces are immersed in a
20 ml vessel in the solution containing the template, the
contents of the vessel is heated to 95°C, cooled slowly over
the period of 10 min to the experimentally predetermined
annealing temperature T_{ann} (T_{ann} can range from room temperature
to 60°C, data not shown). After the annealing step, the
transponders are extensively washed at temperature T_{ann}. The

fluorescence of individual transponders is recorded and the
transponder memories are decoded. Strong fluorescence
indicates that the complement of the dinucleotide sequence
bound to the transponder is present in the target.

EXAMPLE 4

Determination Of The Sequence Of A Three Nucleotide Region
Using A Single-Nucleotide Extension By DNA Polymerase

The target was a single-stranded 50-residue long
oligonucleotide obtained from a PCR reaction after linearly
amplifying DNA. The target DNA does not carry any fluorophore
label. Most of the sequence of 47 nucleotides in the 50 nt
target is known, except for the region of three residues,
residue numbers 18-20, marked as NNN in the figure below:

```
                          5'                    3'

transponder-linker-GTATGGTACTGCAA       oligo   9
              ...-GTATGGTACTGCAC                 8
              ...-GTATGGTACTGCAG                 7
              ...-GTATGGTACTGCAT                 6
              ...-GGTATGGTACTGCA                 5
              ...-GGTATGGTACTGCC                 4
              ...-GGTATGGTACTGCG                 3
              ...-GGTATGGTACTGCT                 2
              ...-CGGTATGGTACTGC                 1
                  | | | | | | | | | | | | | |

3'-ACGTTAAGCCCAGTATGCCATACCATGACGNNNTGGAAGGTAGAGATACT-5'
   ^         ^         ^         ^         ^         ^
   50        40        30        20        10        1
```

The symbol "|" denotes the complementarity of nucleotides in
the two sequences. The linker has the $(dT)_{10}$ sequence to
facilitate annealing. Nine groups of five AVID transponders
are derivatized by the chemical synthesis of oligonucleotides
1 through 9 as shown in the figure above, and electronically
encoded with numbers 1 through 9, respectively. The
transponders are placed in one 5 ml vessel, and 2 mls of the
solution containing the target (final concentration 10 nM to
10 μM) is added to the vessel. The contents of the vessel are

heated to 95°C, cooled slowly over the period of 10 min to the experimentally predetermined extension temperature T_{ex} (T_{ex} can range from room temperature to 60°C, data not shown). After the annealing step, the transponders are extensively washed at

5   temperature T_{ex}. DNA polymerase and four dideoxynucleotide triphosphates, ddATP, ddCTP, ddGTP and ddTTP (obtained from Applied Biosystems) derivatized with four different fluorophores, such as those used in Applied Biosystems automated sequencers are then added to the vessel, and the

10   vessel is incubated at T_{ex} for 15 minutes. Subsequently, the transponders are extensively washed at the wash temperature T_{wash}. A set of data points, each in the form of (oligonucleotide_sequence, fluorescence_intensity, wavelength_of_maximum_fluorescence), is obtained. For highly

15   fluorescent transponders, the wavelength of maximum fluorescence indicates the residue type, and the number encoded on the transponder indicates the position of the residue in the chain, thus defining the sequence of the 3 nt region of the target.